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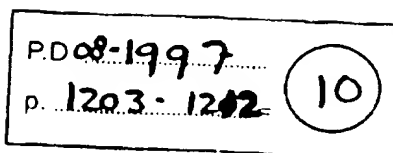
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# DNA vaccination with plasmids encoding the intracellular (HBcAg) or secreted (HBeAg) form of the core protein of hepatitis B virus primes T cell responses to two overlapping K<sup>b</sup>- and K<sup>d</sup>-restricted epitopes

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**Keywords:** hepatitis B core antigen, cytotoxic T lymphocyte, MHC class I, mouse

## Abstract

Plasmid DNA encoding either the intracellular form HBcAg or the secreted form HBeAg of the core protein of hepatitis B virus (HBV) was injected into the muscle of H-2<sup>b</sup>, H-2<sup>d</sup> or F<sub>1</sub><sup>b × d</sup> mice. Serum antibody responses and class I-restricted cytotoxic T lymphocyte (CTL) responses to HBcAg/HBeAg were detected in all mice tested. Stable murine H-2<sup>b</sup> and H-2<sup>d</sup> transfectants that express either intracellular HBcAg or secreted HBeAg were constructed. With these cell lines we re-stimulated *in vitro* T cells primed *in vivo* and detected their specific cytolytic reactivity against naturally processed peptides. CD8<sup>+</sup> CTL responses elicited by DNA vaccination with plasmids encoding HBcAg or HBeAg were specific for the (previously described) K<sup>b</sup>-binding HBcAg<sub>93-100</sub> peptide MGLKFRQL in H-2<sup>b</sup> mice or the (newly defined) K<sup>d</sup>-binding HBcAg<sub>87-95</sub> peptide SYVNTNMGL in H-2<sup>d</sup> mice. The overlapping epitopes span residues 87-100 of HBcAg, and are present on HBcAg and HBeAg. CTL responses were equally well elicited *in vivo* by injecting HBcAg- or HBeAg-expressing plasmid DNA, and CTL efficiently recognize *in vitro* HBcAg- and HBeAg-expressing transfectants. DNA vaccination of F<sub>1</sub><sup>b × d</sup> mice with HBcAg- or HBeAg-expressing plasmid DNA primed CTL populations that recognized the K<sup>b</sup>- or the K<sup>d</sup>-restricted epitope. Both K<sup>b</sup>- and K<sup>d</sup>-binding peptides are thus generated from cytoplasmic/nuclear HBcAg and secreted HBeAg. These data make it unlikely that the appearance of HBeAg-negative variants during chronic HBV infection results from CTL-driven selection. DNA vaccination is an efficient technique to prime CTL responses against overlapping epitopes present on intracellular or secreted viral protein antigens.

## Introduction

MHC class I-restricted cytotoxic T lymphocytes (CTL) protect against many virus infections. CD8<sup>+</sup> CTL specific for different hepatitis B virus (HBV) gene products have been isolated from patients with HBV infection (reviewed in 1). An important immunogenic gene product of HBV is the hepatitis B core antigen (HBcAg). HBc protein is a cytosolic 21 kDa (P21) protein that self-assembles into 34 nm particles which expose HBcAg. The HBc sequence is preceded in-frame by a signal sequence, the so-called pre-core sequence, which converts

HBc protein into the secreted HBe protein. The HBe protein has a translocating signal sequence of 19 amino acids that targets it to the secretory pathway. The P22.5 protein is post-translationally modified in the lumen of the endoplasmic reticulum (ER). Removal of part of the signal sequence and a C-terminal truncation yield the secreted 17 kDa (P17) protein with e antigen activity (HBeAg). In mice class II-restricted T cell epitopes of HBc/eAg have been mapped (2,3). We have recently reported the identification of a class I (K<sup>b</sup>)-restricted

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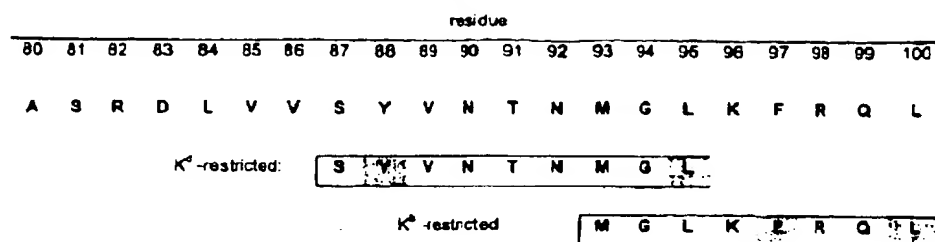


Fig. 1. Location of the K<sup>d</sup> and K<sup>b</sup> epitope in the HBV core protein.

HBc/eAg epitope (4). A therapeutic lipopeptide vaccine based on a human class I-restricted HBcAg epitope has been proposed for the treatment of chronic HBV infection (5).

In DNA vaccination, plasmid DNA is injected and transiently expressed in cells of the skin (6–9) or the muscle (10–12). DNA immunization primes responses against viruses (11–17) and parasites (18,19). Intramuscular inoculation of expression plasmid DNA efficiently primes MHC class I-restricted CTL responses (12,15,18). We have reported the priming of class I-restricted CTL responses to the hepatitis B surface antigen (HBsAg) and to HBc/eAg (4,20–25). We mapped a K<sup>b</sup>-restricted epitope of HBcAg to the residue 93–100 with the sequence MGLKFRQL of the protein (4). Here, we show that DNA vaccination of H-2<sup>d</sup> mice primes CTL responses to the K<sup>d</sup>-binding peptide SYVNTNMGL (residue 87–95) that overlaps the K<sup>b</sup>-binding peptide (Fig. 1). H-2<sup>d</sup> transfectants expressing the intracellular (HBcAg) or the secreted (HBeAg) form of the core protein or targets pulsed with the 9mer peptide SYVNTNMGL were efficiently lysed by CTL from H-2<sup>d</sup> mice vaccinated with expression plasmid DNA encoding either the intracellular HBcAg or the secreted HBeAg. Hence, this epitope can be generated from the cytoplasmic/nuclear and the secreted form of the core protein. In F<sub>1</sub><sup>B<sup>6</sup> × H-2<sup>d</sup></sup> mice, K<sup>b</sup>- as well as the K<sup>d</sup>-restricted CTL reactivities are readily inducible by DNA immunization.

## Methods

### Mice

C57BL/6 J (B6) mice (H-2<sup>b</sup>), BALB/cJ mice (H-2<sup>d</sup>) and F<sub>1</sub> (C57BL/6 × BALB/c) (F<sub>1</sub><sup>B<sup>6</sup> × d</sup>) mice were bred and kept under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). Breeding pairs of these mice were obtained from Bornholtgard (Ry, Denmark). Male and female mice were used at 12–16 weeks of age.

### HBcAg- and HBeAg-encoding expression vectors used for nucleic acid immunization

We have described the construction of the vector pCMV-1 from plasmid pSBC-1 (26), and the insertion of the HBcAg- and HBeAg-encoding genes into pCMV-1 to generate the constructs pCMV-1/c and pCMV-1/e (4). The gene fragment encoding the HBeAg with a mutation of the internal start codon (ATG to ATA) was amplified from a vaccinia recombinant vector kindly provided by H.-J. Schlicht (27) using HBe-specific primers (4). The amplification product was cloned

into the XhoI site of pBluescript II SK<sup>+</sup> to generate pBlue-e<sup>A</sup>. Sequence analysis revealed the expected mutation but no other mutations in the gene. The XhoI fragment from pBlue-e<sup>A</sup> was ligated into *Sac*I-digested pCMV-1 to generate the construct pCMV-1/e<sup>A</sup>. Transient transfection studies demonstrated that HBcAg produced in pCMV-1/c-transfected cells remains largely associated with the cells, while most of the HBeAg produced by pCMV-1/e- and pCMV-1/e<sup>A</sup>-transfected cells was secreted into the supernatant (data not shown). HBeAg expressed from the vector pCMV-1/e<sup>A</sup> was indistinguishable from HBeAg expressed from pCMV-1/e (data not shown).

### HBcAg- and HBeAg-expressing transfectants

The Rauscher virus-transformed T lymphoma line RBL5 (28) is derived from a C57BL/6 (H-2<sup>b</sup>) mouse. This T lymphoma cell line was kindly provided by Dr H.-U. Weltzien (Freiburg, Germany). The H-2<sup>d</sup> mastocytoma cell line P815 (TIB64) was obtained from ATCC (Rockville, MD). Construction of the expression vector pBMG/HBc from the plasmid pBMGneo has been described (4,29). The expression vector pBMG/HBe was generated by insertion of the HBe-containing *Sac*I fragment from pBlue-e into the *Sac*I site of pBMGneo. RBL5 cells or P815 cells were transfected with pBMG/HBe, pBMG/HBc or pBMGneo plasmid DNA.

Expression of intracellular or secreted forms of the core antigen in cell lines was measured in an ELISA using HBcAg, HBeAg-specific antisera. The amount of recombinant HBcAg protein produced in bacteria was determined in SDS-PAGE using BSA as a standard. Serial dilutions of recombinant HBcAg protein were measured in the commercial HBe-MEIA ELISA kit (no. 2227-20; Abbott, Wiesbaden, Germany). The antiserum used in this ELISA cross-reacts with HBcAg and HBeAg. Expression of the intracellular or the secreted form of the core protein by transfectants was measured in arbitrary immunoreactive units (AIU); 10 AIU is the immunoreactive material corresponding to 2 µg recombinant core protein.

### Peptides

Peptides in Table 1 were synthesized in an Applied Biosystems peptide synthesizer model 431A and purified by reverse-phase HPLC. Peptides were dissolved in a 70% acetonitrile/0.1% TFA solution at a concentration of 20 mg/ml and diluted with culture medium for use.

### Nucleic acid immunization

We injected 50 µl PBS containing 1 µg/µl plasmid DNA into each regenerating tibialis anterior muscle 5 days after the

Tabl 1. Fine mapping of the K<sup>d</sup>-restricted epitope of HBcAg

Peptide	Mer	Residue											
		85	86	87	88	89	90	91	92	93	94	95	96
1	12	V	V	S	Y	V	N	T	N	M	G	L	K
2	11		V	S	Y	V	N	T	N	M	G	L	K
3	10			S	Y	V	N	T	N	M	G	L	K
4	9				Y	V	N	T	N	M	G	L	K
5	8					V	N	T	N	M	G	L	K
6	11	V	V	S	Y	V	N	T	N	M	G	L	K
7	10		V	S	Y	V	N	T	N	M	G	L	K
8	9			S	Y	V	N	T	N	M	G	L	K
9	8				Y	V	N	T	N	M	G	L	K
10	7					V	N	T	N	M	G	L	K
11	10	V	V	S	Y	V	N	T	N	M	G	L	K
12	9		V	S	Y	V	N	T	N	M	G	L	K
13	8			S	Y	V	N	T	N	M	G	L	K
14	7				Y	V	N	T	N	M	G	L	K
15	6					V	N	T	N	M	G	L	K

Peptides were derived from the hepatitis B virus core sequence. The amino acid are shown in the usual one-letter abbreviation. The K<sup>d</sup>-specific anchor motif is in bold type. The K<sup>d</sup>-restricted epitope of HBcAg/HBeAg is in italic type.

injection of cardiotoxin (Latoxan, Rosans, France) as previously described (10). The cardiotoxin pretreatment is not critical but enhances CTL priming in most experiments 2- to 4-fold (23). All mice received bilateral intramuscular injections once. Spleen cells were obtained from immunized mice 1-4 weeks post-immunization.

#### Cytotoxicity assay

Spleen cells from immunized or non-immunized (control) mice were suspended in  $\alpha$ -MEM tissue culture medium supplemented with 10 mM HEPES buffer,  $5 \times 10^{-5}$  M 2-mercaptoethanol, antibiotics and 10% v/v FCS (Pan Systems, Aidenbach, Germany). Then,  $3 \times 10^7$  responder cells were co-cultured with  $1.5 \times 10^6$  syngeneic transfectants (irradiated with 20,000 rad) in 10 ml medium in upright 25 cm<sup>2</sup> tissue culture flasks in a humidified atmosphere/7% CO<sub>2</sub> at 37°C. The medium was supplemented with 3% v/v of a rat spleen cell-conditioned medium as a source of growth factors. This conditioned medium was obtained by culturing  $4 \times 10^6$  rat spleen cells/ml for 24 h in medium/5% v/v FCS with 3  $\mu$ g concanavalin A/ml. To determine the restriction specificity of the HBcAg/HBeAg-specific CTL from H-2<sup>d</sup> mice, blocking anti-D<sup>d</sup>, anti-K<sup>d</sup> or anti-L<sup>d</sup> mAb were introduced into the cytotoxic assay in which *in vivo* primed and *in vitro* re-stimulated cytolytic effector cells were co-cultured with transfected targets. The anti-K<sup>d</sup> mAb 31-3-4S (HB77), the anti-D<sup>d</sup> mAb 34-4-21S (HB76) and the anti-L<sup>d</sup> mAb 28-14-8S were used.

Cytotoxic effector populations were harvested after varying intervals of *in vitro* culture and washed twice. Serial dilutions of effector cells were cultured with  $2 \times 10^3$  <sup>51</sup>Cr-labeled targets in 200  $\mu$ l round-bottom wells. Specific cytolytic activity of cells was tested in short-term <sup>51</sup>Cr-release assays against transfected or peptide-pulsed targets and compared with non-transfected/non-pulsed controls. After a 4 h incubation at 37°C, 100  $\mu$ l of supernatant was collected for radiation counting. The percentage specific release was

calculated as  $[(\text{experimental release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})] \times 100$ . Total counts were measured by resuspending target cells. Spontaneously released counts were always <20% of the total counts. Data shown are the mean of triplicate cultures. The SD of triplicates was always <20% of the mean. The data shown were obtained with CTL populations re-stimulated *in vitro* once for 5-7 days. Following repeated *in vitro* re-stimulations of bulk CTL lines, their cytolytic reactivity usually increased.

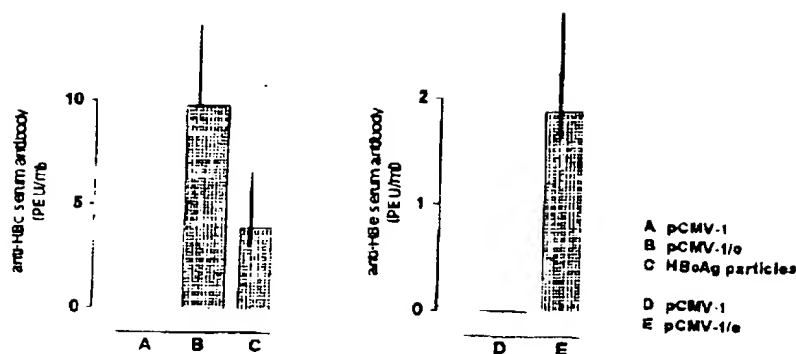
#### Determination of serum antibody levels

Anti-HBc antibodies were detected in mouse sera using the CORE anti-HBc kit (no. 2259-20; Abbott). Concentrations of anti-HBc were standardized against a reference standard of the Paul Ehrlich-Institute (Langen, Germany). Anti-HBe antibody titers in sera of DNA-immunized mice were determined using the IMX anti-HBe assay (Abbott). This assay was standardized using a reference from the Paul Ehrlich Institute.

## Results

#### HBcAg- and HBeAg-expressing transfectants

P815 or RBL5 cells were transfected with DNAs of the vectors pBMG/HBc, pBMG/HBe or pBMGneo (vector DNA with no insert). Stable expression of HBcAg was detected in RBL5/c and P815/c transfectants using a sensitive ELISA (data not shown). In RBL5/c cells HBcAg was present exclusively in cell lysates, but not in supernatants. In P815/c cells, HBcAg was found predominantly in the cell lysate, but low amounts of core antigen were reproducibly found in the supernatant. In pBMG/HBe-transfected P815/e and RBL5/e cells, HBeAg was found predominantly in the supernatant; only low amounts of the secreted form of the core antigen were present in cell lysates (data not shown). Similar data were obtained in immunoprecipitation studies using a polyclonal HBcAg-specific antiserum.



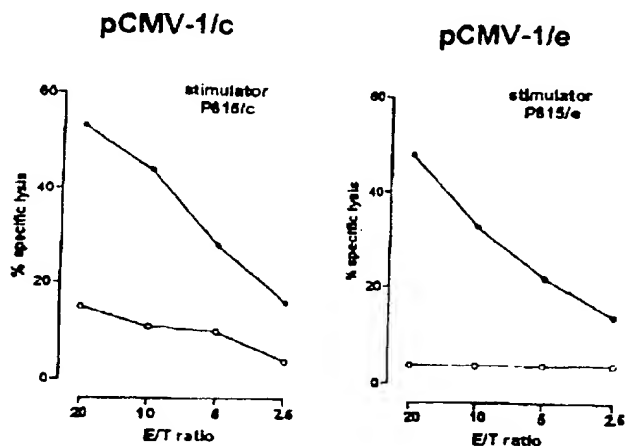
**Fig. 2.** The serum antibody response of H-2<sup>b</sup> mice immunized with pCMV-1/c or pCMV-1/e plasmid DNA. BALB/c mice were immunized by a single intramuscular injection of 50 µg pCMV-1/c (encoding HBcAg) or pCMV-1/e (encoding HBeAg) plasmid DNA or 50 µg pCMV-1 plasmid DNA without insert (none). Mean titers of anti-HBc or anti-HBe antibodies in sera of four mice per group obtained 5 weeks post-immunization are shown. The cut-off point of this assay is 0.1 Paul Ehrlich units/ml.

#### Antibody response of mice immunized with pCMV-1/c or pCMV-1/e plasmid DNA

BALB/c (H-2<sup>d</sup>), C57BL/6 (H-2<sup>b</sup>) or F<sub>1</sub> (BALB/c × C57BL/6) mice were immunized by a single injection of 100 µg pCMV-1/c or pCMV-1/e plasmid DNA into the regenerating muscle. All animals immunized with pCMV-1/c plasmid DNA developed HBc-specific serum antibody titers detectable in an ELISA (Fig. 2B; data not shown). Mice immunized with recombinant HBcAg particles (without adjuvants) also developed HBc-specific serum antibody titers which were always lower than those in DNA-immunized mice (Fig. 2C). Mice immunized with pCMV-1/e plasmid DNA developed anti-HBe serum antibody titers (Fig. 2E). The serum antibody response usually became detectable in the third week post-vaccination and increased for the following 3–6 weeks. In contrast, the CTL response was always detectable from 5 days until >3 months post-vaccination. DNA vaccination of BALB/c, C57BL/6 or F<sub>1</sub> mice with an expression construct encoding either the intracellular form (HBcAg) or the secreted form (HBeAg) of the core protein stimulated the appearance of comparable levels of specific serum antibody titers (4 and data not shown).

#### Immunization of H-2<sup>d</sup> mice with pCMV-1/c or pCMV-1/e plasmid DNA primes *in vivo* K<sup>d</sup>-restricted CD4<sup>+</sup> CD8<sup>+</sup> CTL

As previously reported, intramuscular inoculation of pCMV-1/c or pCMV-1/e plasmid DNA into C57BL/6 (H-2<sup>b</sup>) mice stimulates a K<sup>b</sup>-restricted CTL response to HBcAg/HBeAg (4). We immunized BALB/c mice by an intramuscular injection of 100 µg pCMV-1/c or pCMV-1/e plasmid DNA. Spleen cells obtained from immunized mice 1–6 weeks post-immunization were re-stimulated *in vitro* with transfectants. Cells primed by pCMV-1/c DNA were re-stimulated with P815/c transfectants expressing the intracellular form of the core protein (HBcAg); cells primed by pCMV-1/e DNA were re-stimulated with P815/e transfectants expressing the secreted form of the core protein (HBeAg). *In vivo* primed and *in vitro* re-stimulated CTL populations lysed syngeneic targets expressing the respective antigen (Fig. 3). Spleen cells from unprimed mice (injected with vector DNA pCMV-1 without insert) did not generate a CTL response against HBcAg or HBeAg when



**Fig. 3.** Immunization of H-2<sup>d</sup> mice with pCMV-1/c or pCMV-1/e plasmid DNA primes CTL. BALB/c mice were injected intramuscularly with 100 µg pCMV-1/c or pCMV-1/e plasmid DNA. Spleen cells from immunized mice obtained 10 days post-immunization were re-stimulated *in vitro* for 5 days with inactivated P815/c or P815/e transfectants. The cytotoxic response was tested against HBcAg-expressing P815/c targets or HBeAg-expressing P815/e targets and non-transfected control P815 targets.

co-cultured with syngeneic transfectants (data not shown). Treatment with anti-CD8 antibody plus C', but not treatment with anti-CD4 antibody plus C', eliminated cytolytic effector cells primed to HBcAg or HBeAg by DNA immunization (data not shown). Cytolytic effector cells thus expressed the CD4<sup>+</sup> CD8<sup>+</sup> surface phenotype. *In vivo* priming of CD8<sup>+</sup> CTL precursors by DNA immunization was required to generate cytotoxic effector cells. In mixed lymphocyte tumor culture (MLTC) with mixed (unprimed and primed) CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations, CD4<sup>+</sup> T cells primed *in vivo* by DNA immunization did not support the *in vitro* priming of naive CD8<sup>+</sup> T cells by co-culture with transfectants (data not shown). The anti-K<sup>d</sup> mAb, but not the anti-D<sup>d</sup> or the anti-L<sup>d</sup> mAb blocked recognition of HBcAg-expressing P815/c target cells

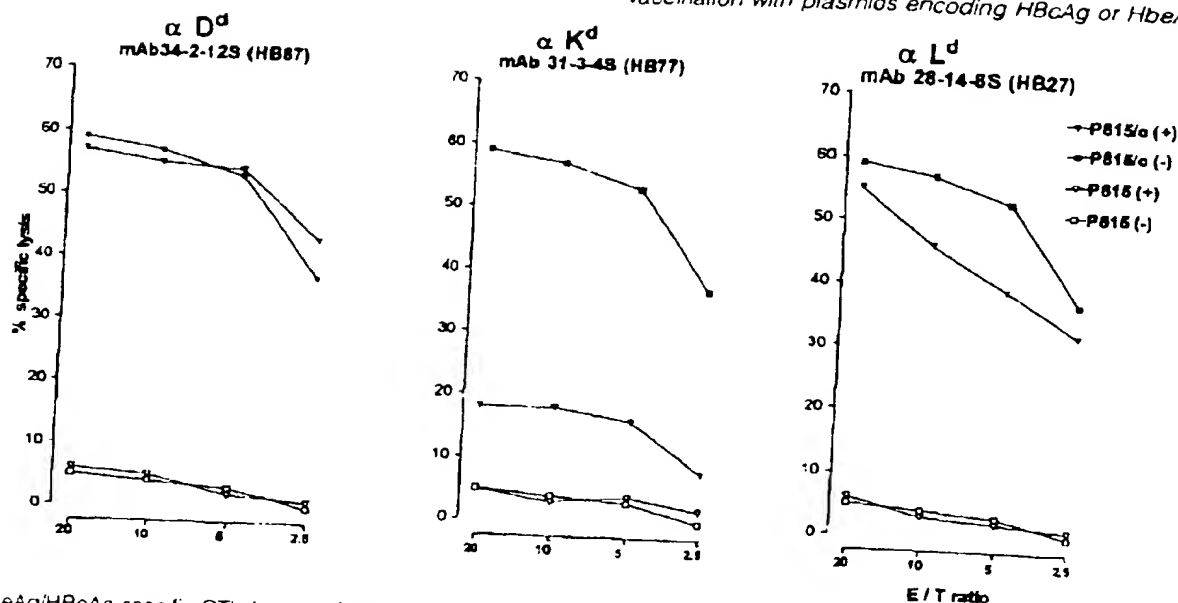


Fig. 4. HBcAg/HBeAg-specific CTL from H-2<sup>d</sup> mice are K<sup>d</sup> restricted. Primed spleen cells from pCMV-1/c-immunized BALB/c mice were co-cultured in the MLTC *in vitro* for 5 days with irradiated P815/c transfectants. The cytotoxic response was read out against P815/c and control P815 targets. The anti-K<sup>d</sup> mAb 31-3-4S (HB77), the anti-D<sup>d</sup> mAb 34-2-12S (HB87) or the anti-L<sup>d</sup> mAb 28-14-8S (HB27) were used. The antibodies were added at a concentration of 5  $\mu$ g/ml (+) or were not added (-) to the medium during the co-culture of effector and target cells.

by HBcAg-primed CTL (Fig. 4). Similar data were found in assays with HBeAg-primed CTL recognizing the HBeAg-expressing P815/e targets (Fig. 4). DNA immunization thus primes *in vivo* CD8<sup>+</sup> CTL precursors that recognize HBcAg/HBeAg-derived peptide(s) in the context of K<sup>d</sup>.

#### HBcAg/HBeAg-specific CTL from H-2<sup>d</sup> mice recognize the K<sup>d</sup>-restricted epitope SYVNTNMGL

A library of overlapping synthetic peptides of the HBV core antigen was used to map the epitope recognized by CTL from pCMV-1/c or pCMV-1/e immunized H-2<sup>d</sup> mice (4). Only P815 cells pulsed with 10<sup>-6</sup> M of a peptide representing residues 82-101 of HBcAg were lysed by short-time CTL lines (12-25% specific lysis at an effector:target ratio of 40). Fine mapping of the epitope identified it as the 9mer peptide SYVNTNMGL spanning residues 87-95 of HBcAg (Table 1 and Fig. 5). Similar data were obtained when CTL lines from mice immunized by pCMV-1/c or pCMV-1/e and stimulated *in vitro* with P815/c or P815/e cells were tested. Immunization of mice with HBeAg- or HBcAg-encoding plasmid DNA thus generated CTL with this epitope specificity. The K<sup>d</sup>-restricted HBcAg epitope contains the K<sup>d</sup> allele-specific motif, with Y at position 2 and L at position 9 (30). The sequence of the 9mer peptide recognized in the context of K<sup>d</sup> overlaps by three residues the sequence of the 8mer epitope of HBcAg recognized in the context of K<sup>b</sup> (Fig. 1).

#### Priming *in vivo* and re-stimulation *in vitro* of HBcAg/HBeAg-specific CTL with constructs/transfectants expressing either the intracellular or the secreted form of the core protein

We primed H-2<sup>d</sup> mice with pCMV-1/c or pCMV-1/e plasmid DNA. Primed cells were re-stimulated in the MLTC *in vitro*

either with HBcAg-expressing P815/c transfectants or with HBeAg-expressing P815/e transfectants. The cytotoxic effector cells were tested against either P815/c targets or P815/e targets. All CTL populations primed and re-stimulated by HBcAg or HBeAg displayed K<sup>d</sup>-restricted specificity for the HBcAg<sub>87-95</sub> epitope. No differences in the efficiency of priming *in vivo*, re-stimulation *in vitro* or target cell recognition *in vitro* were detectable in five independent experiments (Fig. 6 and data not shown). The amount of DNA used in vaccination was titrated by injecting 100, 20 or 4  $\mu$ g/mouse (Table 2). These data confirmed that constructs encoding the intracellular form (HBcAg) or the secreted (HBeAg) form of the core antigen elicited CTL responses when 100 or 20  $\mu$ g of the plasmid DNA were injected. Similar data were obtained when pCMV-1/e or pCMV-1/e<sup>+</sup> vector DNA were injected. Hence, processing and/or presentation of the K<sup>d</sup>-binding peptide from cytoplasmic/nuclear HBcAg or secreted HBeAg was apparently of comparable efficiency in different cell types.

#### In DNA-immunized F<sub>1</sub><sup>bxd</sup> mice CTL populations specific for the K<sup>b</sup> or the K<sup>d</sup>-restricted epitope are co-primed

The K<sup>b</sup>-restricted 8mer HBcAg<sub>93-100</sub> epitope MGLKFRQL and the K<sup>d</sup>-restricted 9mer HBcAg<sub>87-95</sub> epitope SYVNTNMGL overlap (Fig. 1). We tested if two CTL populations specific for either the K<sup>d</sup>-restricted epitope or the K<sup>b</sup>-restricted epitope are co-primed in F<sub>1</sub><sup>bxd</sup> mice by pCMV-1/e or pCMV-1/c DNA immunization. The data shown in Fig. 7(E and F) demonstrate that both CTL populations are readily detectable in DNA vaccinated F<sub>1</sub> mice. The magnitude of the CTL response of F<sub>1</sub> mice against the K<sup>d</sup>- and the K<sup>b</sup>-restricted epitope was comparable to the CTL response of H-2<sup>b</sup> mice against the K<sup>b</sup>-restricted epitope (Fig. 7A and B) and of H-2<sup>d</sup> mice against

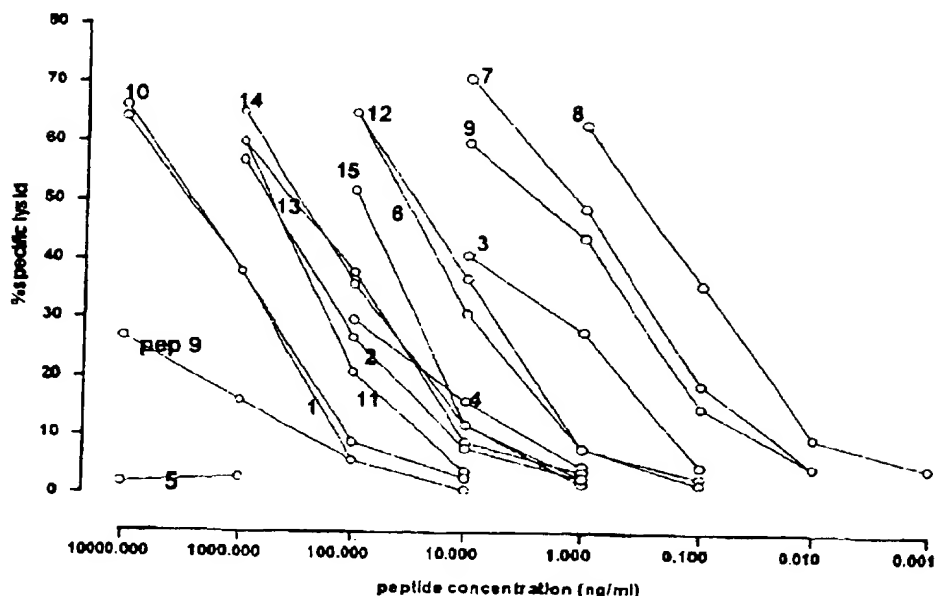


Fig. 5. Mapping of the  $K^b$ -restricted epitope of HBcAg/HBeAg. Splenic CTL populations from BALB/c mice primed by pCMV-1/c plasmid DNA immunization were re-stimulated *in vitro* with P815/c transfectants and used for peptide mapping. Radiolabeled P815 were pulsed with the indicated concentration of peptides for 2 h at 28°C, washed and used as targets. Designation of the peptides is that listed in Table 1.

the  $K^d$ -restricted epitope (Fig. 7C and D). DNA vaccination of  $F_1$  mice thus primes distinct CTL populations with different restriction specificities that recognize overlapping peptides efficiently processed from an intracellular or a secreted protein antigen.

## Discussion

### Potent stimulation of CTL responses by DNA immunization

The study confirms that DNA vaccination is a potent technique to stimulate class I-restricted T cell responses to viral antigens. DNA vaccination revealed  $K^b$ - and  $K^d$ -restricted CTL responses to HBcAg in mice. CTL responses were not primed in H-2<sup>b</sup> or H-2<sup>d</sup> mice by injections of recombinant HBcAg core particles by different routes. This is in contrast to other 'virus-like particle' systems in which CTL responses can be primed by injecting low doses of lipoprotein particles without adjuvants. This has been shown in the HBsAg system (20–22, 24, 31–34), in the HIV gag system (35) and in the Ty 'virus-like particle' system (36, 37). It is unknown why HBcAg particles have only a low immunogenicity for CTL precursors.

In some antigen systems induction of a class I-restricted CTL response may be 'difficult'. Although CTL can recognize peptide epitopes of these antigens binding to MHC class I molecules with high affinity and can mediate different effector functions *in vivo*, they are difficult to prime with traditional vaccination approaches. We have described this with the HBsAg in H-2<sup>b</sup> mice (25) and with the SV40 T antigen in H-2<sup>d</sup> mice (38). Once induced, such T cell response may have a considerable prophylactic or therapeutic potential. This observation makes DNA vaccination an interesting option in the immunotherapy of cancer and persisting infections.

### Natural processing and presentation of two overlapping class I-restricted epitopes

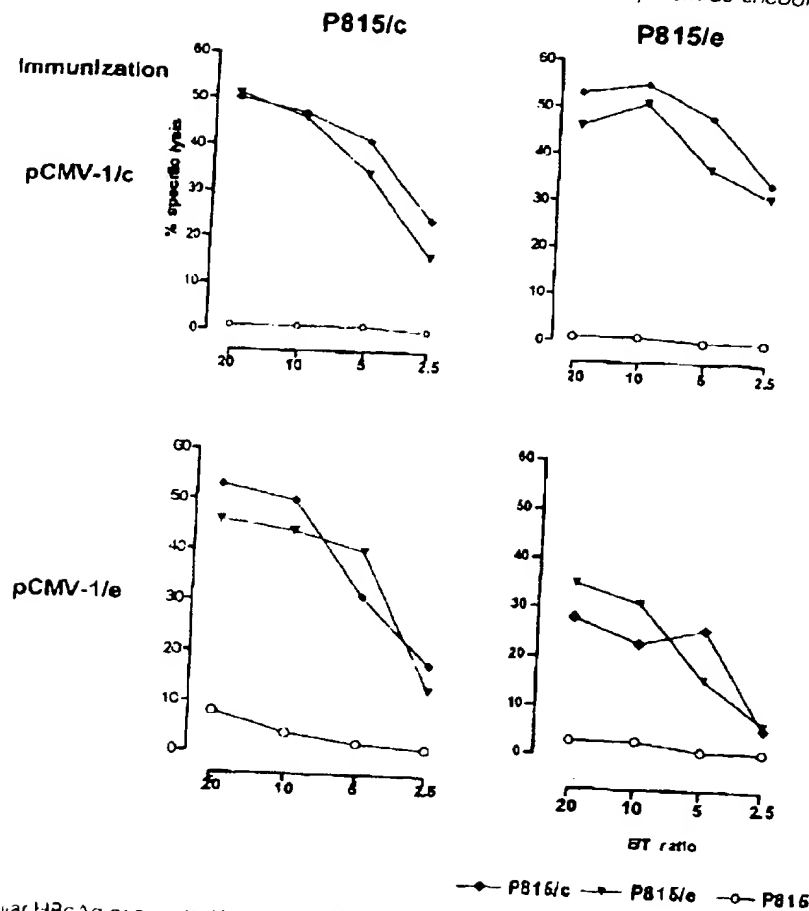
DNA immunization of C57BL/6 (H-2<sup>b</sup>) mice elicits  $K^b$ -restricted CTL responses against the 9mer epitope MGLKFRQL (HBcAg<sub>93–100</sub>) of HBcAg/HBeAg (4). This epitope stimulates a CTL response when mice are immunized with HBcAg- or with HBeAg-encoding expression plasmids. This epitope is generated during natural processing of the secreted form (HBeAg) and the cytoplasmic form (HBcAg) of the core antigen. The 8mer epitope is of the expected length, contains the  $K^b$ -specific anchor motif (30) and binds to  $K^b$  with high affinity. It resembles neither the HLA class I-restricted epitopes of HBcAg reported so far (1) nor the murine class II-restricted epitopes of HBcAg/HBeAg (2, 3).

In this paper, we have mapped the  $K^d$ -restricted 9mer HBcAg<sub>87–95</sub> peptide SYVNTNMGL as a further CTL epitope. It overlaps with the  $K^b$ -restricted epitope by three amino acid residues. The efficiency of antigen processing seems to depend on intrinsic qualities of an epitope and its location within the protein. Proteolytic degradation of HBcAg/HBeAg to generate these epitopes may involve one or multiple steps. Processing could generate a 14mer (or larger) peptide, that is transported through the TAP, binds to MHC class I molecules and is subsequently trimmed to the appropriate size. Alternatively, different proteolytic cleavages could generate two different peptides that each contains one of the two epitopes.

### Secreted and intracellular forms of the HBV core protein are efficiently processed for class I-restricted presentation

The pre-core region of HBV contains two alternative ATG translation initiation codons. The precursor for the secreted P22 S form of the core protein is translated from the first ATG;





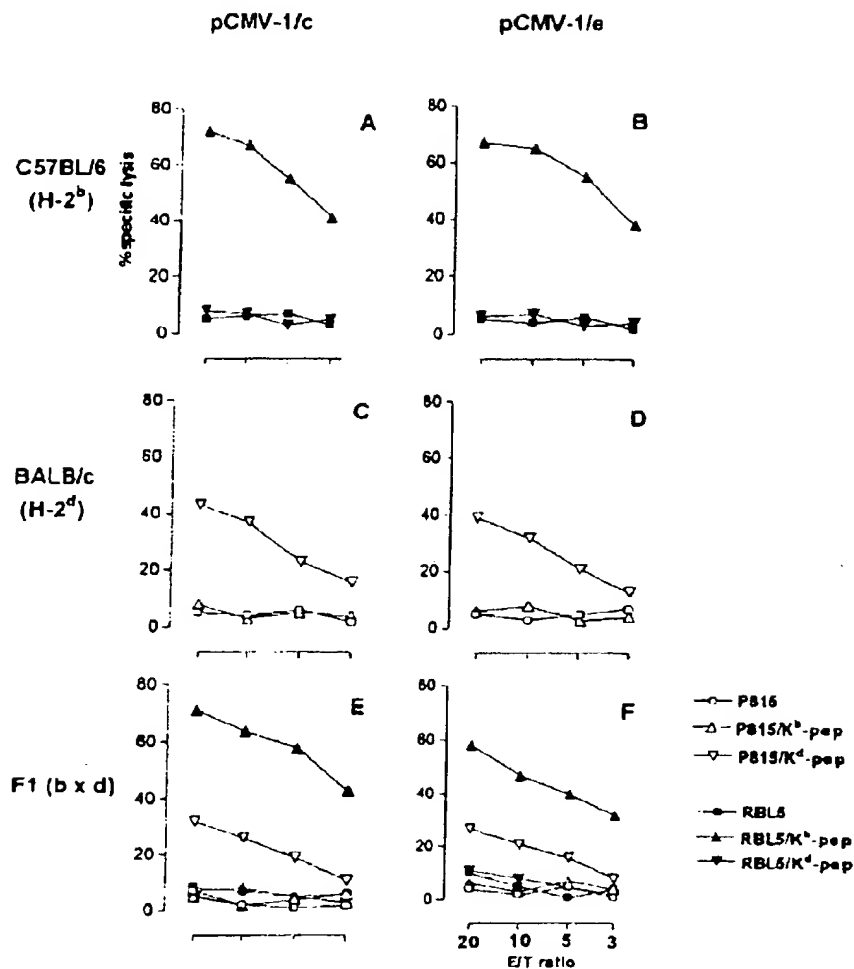
**Fig. 8.** Processing of intracellular HBcAg or secreted HBeAg leads to efficient K<sup>d</sup>-restricted epitope presentation. BALB/c mice were immunized with HBcAg-expressing pCMV-1/c plasmid DNA or with HBeAg-expressing pCMV-1/e plasmid DNA. Primed cells were re-stimulated *in vitro* either with HBcAg-expressing P815/c transfectants or with HBeAg-expressing P815/e transfectants. The cytotoxic response was tested in a 4h <sup>51</sup>Cr-release assay either against HBcAg-expressing P815/c targets or HBeAg-expressing P815/e targets.

**Table 2.** Induction of CTL responses by injecting titrated amounts of plasmid DNA

Plasmid <sup>a</sup>	Dose (µg/mouse)	CTL response <sup>b</sup>							
		P815/c				P815			
		20	10	5	2.5	20	10	5	2.5
pCMV-1/c	100	29	29	23	16	8	5	2	2
	20	19	13	12	6	11	7	5	3
	4	1	0	1	2	1	1	0	0
pCMV-1/e	100	33	26	20	18	8	3	3	0
	20	32	26	23	15	6	5	2	3
	4	17	15	8	7	14	9	5	3
pCMV-1/e <sup>Δ</sup>	100	29	21	18	13	4	1	0	4
	20	21	15	7	5	5	4	5	4
	4	29	19	15	8	5	2	4	2

<sup>a</sup>Titred amounts of expression plasmid DNA encoding the intracellular (pCMV-1/c) or the secreted (pCMV-1/e or pCMV-1/e<sup>Δ</sup>) form of the core protein were injected intramuscularly into BALB/c mice.

<sup>b</sup>The cytotoxic response was read out against P815/c targets or P815 targets at the indicated effector:target ratios.



**Fig. 7.** K<sup>d</sup>- and K<sup>b</sup>-restricted CTL are co-primed by immunizing F<sub>1</sub><sup>B6xJ</sup> mice against HBcAg or HBeAg by DNA vaccination. C57BL/6 (H-2<sup>b</sup>) (A and B), BALB/c (H-2<sup>d</sup>) (C and D) or F<sub>1</sub><sup>B6xJ</sup> (BALB/c x C57BL/6) (E and F) mice were immunized with pCMV-1/c (A, C and E) or pCMV-1/e (B, D and F) plasmid DNA. Primed spleen cells obtained 2 weeks post-immunization were specifically re-stimulated for 5 days in the MLTC: cells from C57BL/6 mice were re-stimulated with inactivated RBL5/c transfectants; cells from BALB/c mice were re-stimulated with inactivated P815/c transfectants and cells from F<sub>1</sub> mice were re-stimulated with a mixture of inactivated RBL5/c and P815/c transfectants. Similar data were obtained when primed F<sub>1</sub> cells were re-stimulated *in vitro* either with RBL5/c or with P815/c cells or with peptide-pulsed stimulator cells. The specific cytolytic reactivity of CTL was read out against: P815 cells pulsed with the K<sup>b</sup>-binding HBcAg<sub>93-100</sub> peptide MGLKFRQL (P815/K<sup>b</sup>-pep) or the K<sup>d</sup>-binding HBcAg<sub>57-65</sub> peptide SYVNTNMGL (P815/K<sup>d</sup>-pep). RBL5 cells pulsed with the K<sup>b</sup>-binding HBcAg<sub>93-100</sub> peptide MGLKFRQL (RBL5/K<sup>b</sup>-pep) or the K<sup>d</sup>-binding HBcAg<sub>67-75</sub> peptide SYVNTNMGL (RBL5/K<sup>d</sup>-pep), or non-treated P815 or RBL5 control targets.

the intracellular form of the core protein is translated from the second ATG. To exclude the possibility that low levels of HBcAg are translated from the second ATG during biosynthesis of P22.5 from the first ATG, we mutated the second ATG. From this construct (pCMV-1/e<sup>3</sup>), only secreted HBeAg can be expressed. Injection of this plasmid primed K<sup>b</sup>- and K<sup>d</sup>-restricted CTL to HBcAg efficiently (Table 2). The secreted form of the core antigen can therefore be processed for class I-restricted peptide presentation.

We observed no differences in the efficiency of CTL priming by plasmid DNA encoding HBeAg or HBcAg and we found no differences in the efficiency of K<sup>b</sup>- or K<sup>d</sup>-restricted presentation of the HBcAg/HBeAg epitopes by transfectants. These

data indicate that generation of peptides from secreted and intracellular proteins for loading to MHC class I molecules is equally efficient. The site of processing of secreted HBeAg, and the site of loading of the generated peptides to K<sup>b</sup> and K<sup>d</sup> molecules, remains to be elucidated. Cytosolic degradation of proteins seems to be the major source for peptides presented by MHC class I molecules (39). The site of processing of secreted proteins for recognition by CTL is uncertain. Most secreted proteins, including the P22.5 protein, are co-translationally translocated into the ER lumen during biosynthesis. In peptide transporter (TAP)-deficient cell lines, processing within the ER lumen has been shown to generate MHC class I-binding peptides, mostly derived from cleaved

leader sequences (40). A retrograde transport of polypeptides or peptides from the ER lumen into the cytosol may feed them into a pool that is available for TAP-dependent loading on nascent class I molecules (41,42). Low-frequency failure of the signal sequence-containing polypeptide to engage the translocation apparatus, resulting in synthesis and degradation of the protein in the cytosol, may represent an important mechanism for the generation of class I-restricted CTL responses (43,44). Secreted exogenous proteins may be processed in alternative pathways to generate peptides available for MHC class I molecule loading (reviewed in 45,46).

HBeAg-negative variants often arise in the course of chronic HBV infections. These HBeAg-non-expressing HBV variants often carry a stop codon at residue 28 encoding the leader sequence or contain core promoter mutations (47,48). Our data make it unlikely that the appearance of HBeAg-negative variants during chronic HBV infections results from CTL-mediated immune selection. Expression of the secreted and the intracellular form of the core antigen was equally efficient in (re)stimulating murine CTL responses. Observations in patients support these findings: HBcAg-specific CTL from acutely HBV-infected patients lyse targets equally well that express either HBcAg or HBeAg (1,49,50). The only domain of the secreted form of the core antigen that could stimulate CTL operating in the immune selection of HBeAg-negative variants would be epitopes in the HBeAg leader sequence; such CTL reactivity has not been observed in chronically infected patients (1). Cells infected with a HBeAg-negative HBV variant will be visible to HBcAg-specific CTL and will therefore not be positively selected. Furthermore, CTL are unlikely to exert a strong selection pressure for HBeAg-negative variants of HBV because they are very difficult to detect in chronically infected patients. These data make it unlikely that the appearance of HBeAg-negative variants during chronic HBV infection is the result of CTL-mediated immune selection.

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### Abbreviations

AIU	arbitrary immunoreactive unit
AFC	antigen-presenting cell
CTL	cytotoxic T lymphocyte
ER	endoplasmic reticulum
HBcAg	intracellular form of hepatitis B core antigen
HBeAg	secreted form of hepatitis B core antigen
HBV	hepatitis B virus

MLTC	mixed lymphocyte tumor cell culture
TAP	transporter associated with antigen processing

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